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Gametophyte Development, Sex Expression and Antheridiogen System in *Pteris incompleta* Cav. (Pteridaceae)

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Abstract.—The gametophytic phase of several species of *Pteris* has been well studied, but for others, due perhaps to their more restricted distribution, little is known. Agar and soil cultures of different spore samples of *P. incompleta* were established in order to analyze developmental features of its gametophytes. Gametophyte development followed the *Ceratopteris* pattern, but resulted in a slightly different morphology from that of other more common species of the genus. Sex expression was variable among gametophyte populations, and was affected by culture medium. An antheridiogen system was present and promoted both male precocity and dark germination. Antheridiogen response was variable among gametophyte populations. Positive antheridiogen response in interspecific gametophyte pairings suggests a common antheridiogen system in *Pteris vittata* and *P. incompleta*.

Key Words.—Gametophyte, morphology, sexual expression, antheridiogen, *Pteris incompleta*

Pteris is a large and diverse genus of about 250 terrestrial and epilithic species (Tryon and Tryon, 1982) of predominantly tropical and subtropical distribution. Despite being one of the largest genera of homosporous ferns, little is known about the sexual generation of *Pteris* species (Atkinson, 1973; Laird and Sheffield, 1986; Chiou, 1992; Mendoza *et al.*, 1996–97) except for *P. vittata* L. which has been studied from many morphological and physiological aspects (Ito, 1962; Kato, 1963; Kato, 1969; Gemmrich, 1986 a–b; Tu and Ma, 2003).

Only a few species of *Pteris* grow in temperate areas; one of them, *P. incompleta* Cav., is native to the Macaronesian and Mediterranean regions where it represents a relic of a Tertiary flora that virtually disappeared with the first glaciations (Pichi Sermolli, 1979; Salvo, 1990). It is a protected species in Spain where its populations are, at present, very scarce and vulnerable, in great part because of the fragility of the ecosystems where the species grows.

Our goal was to study gametophyte development and sex expression of *P. incompleta* under laboratory conditions. Some of our preliminary results suggested the possibility of an antheridiogen system operating in this species. An antheridiogen system similar to that of *Pteridium aquilinum* (L.) Kuhn, which induces the formation of antheridia in young prothalli and substitutes for the light requirement in spore germination, is known to be produced by *P. vittata* (Gemmrich, 1986 a–b; Raghavan, 1989). This antheridiogen is active in

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TABLE 1. List of the different cultures for antheridiogen assays in light conditions, depending on the combination of samples used as source of female gametophytes / spores. Cultures marked with an asterisk were also established for assays in the dark. VA: *P. vittata* (Valencia); CA: *P. incompleta* (Cádiz); GC: *P. incompleta* (Gran Canaria); GO: *P. incompleta* (La Gomera).

Culture n°	GF ♀	Spores
1	-	VA
2	VA	VA
3	CA	VA
4*	GC	VA
5	-	GC
6	CA	GC
7	GC	GC
8	-	GO
9*	GO	GO
10*	VA	GO

other species of *Pteris* and in different fern genera (Schneller, 1979; Gemmrich, 1986b; Schneller *et al.*, 1990; Nester-Hudson *et al.*, 1997; Chiou and Farrar, 1997). Therefore additional assays were undertaken to test this hypothesis.

MATERIALS AND METHODS

Spores used for this study were taken from sporophytes collected in the locations listed in Table 1. Voucher specimens are deposited in the Universidad Complutense de Madrid – Biología herbarium (MACB).

Spore samples for cultures were taken from single sporophytes kept dry at room temperature since the plants were collected. Gametophytes were grown under fluorescent light on a 12-h light, 12-h dark cycle at 20 ± 2°C, in plastic Petri dishes 6 cm in diameter.

Spore germination and gametophyte development.—Multispore cultures on mineral agar (medium described in Dyer, 1979) were established by sacking fertile pinnae on a piece of weigh paper and placing the obtained spores in the Petri dishes. Sowing of each sample was replicated twice. Percentage germination was recorded for a random sample of 50 spores from each of two plates, every three days until there was no further increase.

To study the different represented stages of development from spore germination until sexual maturity (which lasted ca. 9 weeks), random samples were taken weekly. Gametophytes were stained with chloral hydrate acetocarmine (Edwards and Miller, 1972), mounted in water and observed under a light microscope. These agar cultures were used also as a source of young presexual gametophytes in transplant studies of sex expression on soil cultures.

Sex expression.—Cultures on soil (a 3:1 mixture of commercial compost and sand) were used to study sexual expression of samples GC, CA and VA (see Table 1). In order to observe the sequence of formation of gametangia on each individual gametophyte, 50 young gametophytes from the multispore agar cultures were transplanted at the 15–30 cell stage and separately arranged in a

TABLE 2. Percentages of spore germination for each sample, time required for the beginning of germination and, in brackets, weeks at which those percentages were reached. The column "age" shows the time passed from collecting the fronds to spore sowing.

SAMPLE	AGE	TIME FOR GERMINATION	% OF GERMINATION
P. incompleta GC	60 weeks	2 weeks	45 (8)
P. incompleta CA	24 weeks	3 weeks	100 (4)
P. incompleta GO	17 weeks	1 week	87 (3)
P. vittata VA	1 week	1 week	100 (1)

regular pattern in two Petri dishes as follows: six rows of gametophytes per dish, the first row with two gametophytes, the following four rows with five gametophytes per row, and the last row with three. The final density was ca. one prothallus cm^{-2}. Examinations were made at two week intervals for 18 weeks. For these observations, gametophytes were individually mounted in water, examined with a compound microscope and replaced back into culture.

Multispore cultures on mineral agar were also used for samples GC and VA in order to test if sex expression is affected by culture medium. Random samples of ca. 50 gametophytes from both cultures were taken at two-week intervals during the 18 weeks, stained as described above and the percentages of sterile, male, female and bisexual gametophytes calculated.

Antheridiogen assays.—To test antheridiogen activity in *P. incompleta*, two different aspects were studied: induction of male precocity and induction of spore germination in darkness. Several tests were made using *P. incompleta* and *P. vittata*. *Pteris vittata* was used as reference organism because its antheridiogen system has been described and its reactions to antheridiogen are well known (Gemmrich, 1986a–b; Raghavan, 1989). *Pteris vittata* was used in two ways: a) as a source of antheridiogen to test antheridiogen response in spores and gametophytes of *P. incompleta*; and b) as a bioassay to measure response to antheridiogen activity of female *P. incompleta* gametophytes.

As we had access to several samples of *P. incompleta*, multiple combinations of female gametophytes/spores were arranged as is shown in Table 1. All the cultures were established on mineral agar, as described above for spore germination, placing one female gametophyte in the center of the Petri dish and sowing the corresponding spores around it. Controls for the antheridiogen response of both species were made in similar cultures without female gametophytes (Table 1, cultures 1, 5 and 8).

To detect male precocity, random samples of ca. 50 gametophytes of each culture were taken at intervals of two weeks during 18 weeks, stained as described above for spore germination and percentages of sterile, male, female and bisexual gametophytes were calculated (Table 1, cultures 2, 3, 4 ,6, 7, 9 and 10) .

To carry out the test of spore germination in the dark, the combinations of samples of cultures 4, 9 and 10 (Table 1) were used. Six replicates were made for each culture, three with only spores as control, and three with a female gametophyte and the corresponding spores around it. Plates were wrapped in

two folds of aluminium paper, kept in a box and placed in the same growth room with the other cultures. Each pair of plates (with and without female gametophyte) was unwrapped and the content examined after 2, 4 and 6 weeks.

Results

Spore germination and gametophyte morphology.—Spore germination in *Pteris incompleta* is of the *Vittaria* type, the most common in homosporous ferns (Nayar and Kaur, 1971). Table 2 shows rates of spore germination obtained for each sample, as well as the time passed from sowing to the beginning of germination, which was evident when the first rhizoid emerged.

After spore germination, the first rhizoid and a short filament of 1–4 cells were formed. The filament divides its apical or subapical cell starting to produce a narrow plate and later a spatulate one, following the *Ceratopteris* type of development described by Nayar and Kaur (1971). The plate lacks a meristem at this first stage, but for most of the prothalli the meristem is organized after 1 to 3 weeks of growth following germination.

The meristem is in an apical or slightly lateral position. Activity of meristematic cells gives rise after a few days to a cordate but somewhat asymmetrical prothallus with two wings of almost the same size (Fig. 1a–f). By the time that many of the gametophytes reached the cordate shape other gametophytes of the cultures remained irregular, with or without a defined meristem. Mature gametophytes were naked (without hairs), either cordate (Fig. 1g–h) or irregular (Fig. 1i).

Sex expression.—Progression of sex expression on soil cultures for the samples studied of *P. incompleta* (GC and CA) and *P. vittata* (VA) is summarized in Figure 2. Sample GC showed a small proportion (12%) of female cordate prothalli 8 weeks after sowing, the remaining being sterile. Two weeks later male gametophytes were abundant (62%) and a moderate proportion (28%) of sterile became female. Male gametophytes were small, irregular, and without a defined meristem. When cultures were 18 weeks old, bisexual gametophytes were produced (24%), most of them developing from the males, in which a meristem differentiated and archegonia formed near the meristem area (Fig. 2c). Bisexual gametophytes were cordate but smaller than the females.

Sample CA showed simultaneously cordate male and female gametophytes 8 weeks after sowing in a proportion of 42% and 46% respectively; only 4% were sterile by that time and the remaining 8% were cordate female gametophytes from which an irregular basal lobe was formed and produced antheridia, then such gametophytes became functionally bisexual. At 10 weeks the few sterile prothalli became male and some females formed the lobes with antheridia. The proportions of each sexual type had not change at 18 weeks (Fig. 2e).

In *P. vittata* 78% of gametophytes were female, 12% bisexual and only the remaining 10% presexual by week 8. Two weeks later presexual gametophytes and most of the females became bisexual, and only 6% remained female.

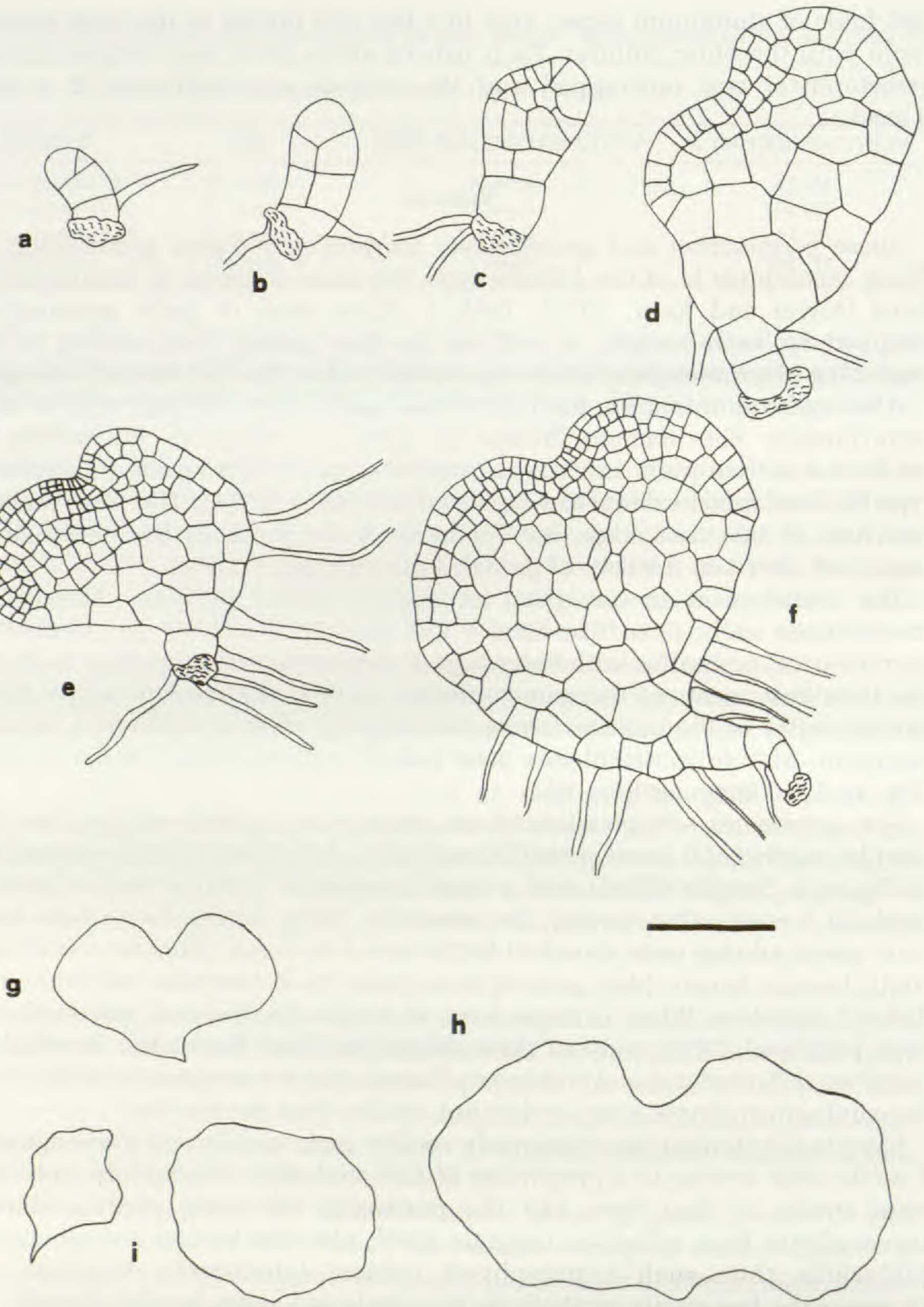

FIG. 1. Gametophyte development in *P. incompleta*. a–f. Early stages showing the asymmetrical shape in young prothalli; g. Presexual cordate gametophyte; h. Mature female prothallus; i. Irregular male prothalli. Scale bar: a–d, 50 μm; e–f, 100 μm; g–i, 1000 μm.

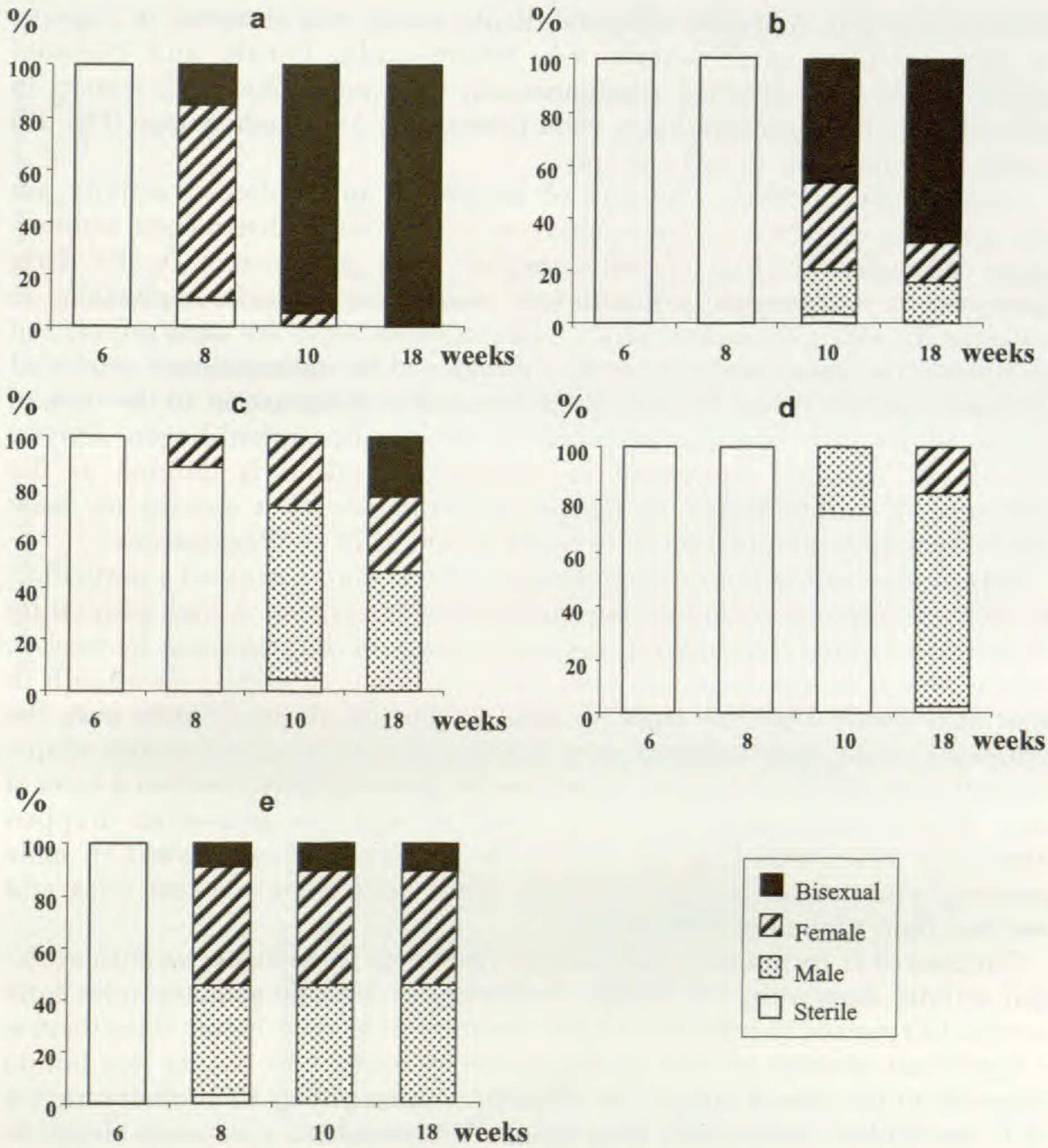

FIG. 2. Progression of sex expression of *Pteris* (percentage of gametophytes by sex / weeks after sowing). a–b. *P. vittata*; c–d. *P. incompleta* sample GC; e. *P. incompleta* sample CA; a, c and e. Soil cultures; b and e. Agar cultures.

Progression of sex expression in the following weeks involved the transformation of females into bisexuals by production of antheridia near the apical notch where archegonia were placed, so that by week 18 100% of gametophytes were bisexual (Fig. 2a).

Progression of sex expression was also observed in the cultures on agar of samples GC and VA (Table 1, cultures 5 and 1, respectively). In sample GC only male and female gametophytes were detected over the same period of observation; the first gametangia produced were antheridia instead of archegonia and their formation started two weeks later with respect to soil

cultures (Fig. 2d). A similar temporal displacement was observed in cultures on agar medium of *P. vittata* VA, where male, female and bisexual gametophytes were detected simultaneously two weeks later with respect to soil cultures; male gametophytes were detected in a low percentage (Fig. 2b) which were not seen in soil cultures.

Antheridiogen activity.—Results of assays on antheridiogen activity are segregated in response to endogenous or exogenous antheridiogen sources, tested through induction of male precocity and germination in the dark. Response to endogenous antheridiogen (which we consider equivalent to intraspecific effect, the gametophytic individuals being of the same population or different) is considered here as the influence of the antheridiogen produced by female gametophytes on male precocity and/or germination in the dark of spores of its own species. Response to exogenous antheridiogen activity (which we consider equivalent to interspecific effect) is defined as the influence of antheridiogen of female gametophytes of a species on male precocity and/or germination in the dark of spores of another species.

Response to endogenous antheridiogen.—*Pteris vittata* showed a particularly vigorous response to endogenous antheridiogen activity. A high proportion of prothalli formed antheridia in very early stages of development: by week 6, nearly 50% of the gametophytes were male, spatulate, ameristic, 40–50 cells in size; and nearly 90% by week 8; this proportion changed little over the following weeks; gametophytes grew slowly and never reached cordate shape. Control cultures, those without added female gametophytes, reached a level of only 50% of male gametophytes by week 14, and this proportion dropped drastically afterwards (Fig. 3a) due to the formation of archegonia in male gametophytes which became bisexual, grew more than the last ones and reached their normal cordate shape.

Cultures of *P. incompleta* had variable responses to endogenous antheridiogen activity depending on sample combinations. Female gametophytes from sample GO seemed to induce a middle response in its own spores since there is a significant increase of male gametophytes by weeks 8 to 10 (Fig. 3c), but no response in the case of sample GC (Fig. 3b). Gametophytes from other samples of *P. incompleta*, particularly from sample CA, promoted a response almost as high as in the case of *P. vittata* (Fig. 3b).

Germination in the dark due to endogenous antheridiogen activity was tested in *P. incompleta* GO spores sown with female gametophytes of the same sample. A low percentage (3%) of spores germinated by week 6. Those gametophytes consisted of short, curved uniseriate filaments of 2–3 cells, some bearing antheridia. In control cultures, those sown in the dark without female gametophytes, there was no germination.

Response to exogenous antheridiogen.—*Pteris vittata* was greatly influenced by the antheridiogen of *P. incompleta*, from both samples CA and GC: gametophytes formed antheridia more than 4 weeks before control cultures, and the proportion of male prothalli rapidly reached nearly 100%. In the CA/VA combination, 22% of the gametophytes bore antheridia after only two weeks in culture (Figs. 3d, 4a).

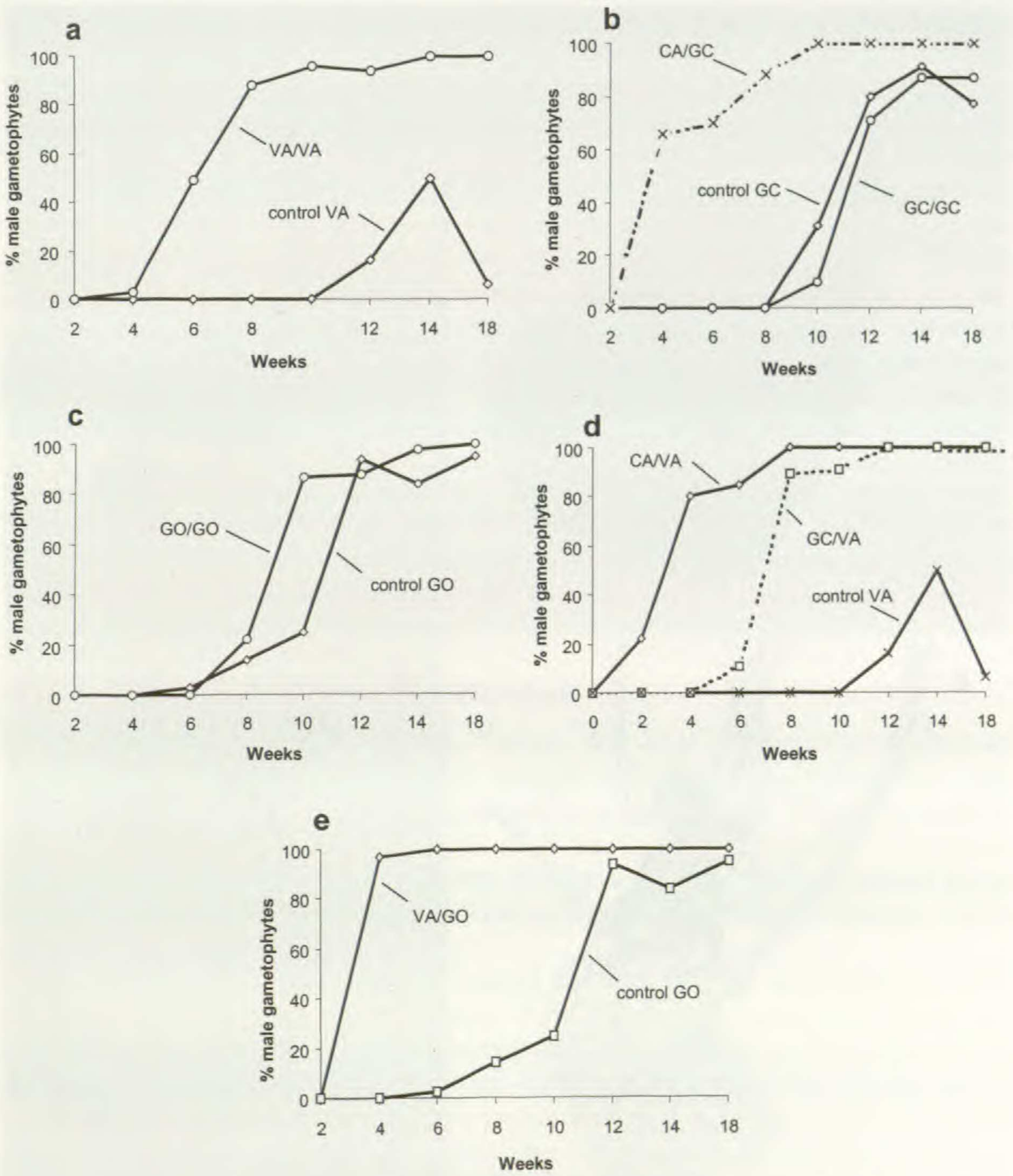

FIG. 3. Comparison between control tests and the corresponding cultures for antheridiogen response, indicated as pairs of samples: source of female gametophyte / source of spores. VA, *Pteris vittata*; CA, GC and GO, *P. incompleta*.

The reverse situation was detected also: *P. incompleta* was influenced by the antheridiogen of female gametophytes of *P. vittata*. As Fig. 3e shows, 4 weeks after sowing near 100% of gametophytes in sample GO developed antheridia (Fig. 4c); this proportion did not change in the following 14 weeks. This strong maleness is precocious since the first male gametophytes appeared in the control culture four weeks later and in a lower initial percentage (Fig. 3e).

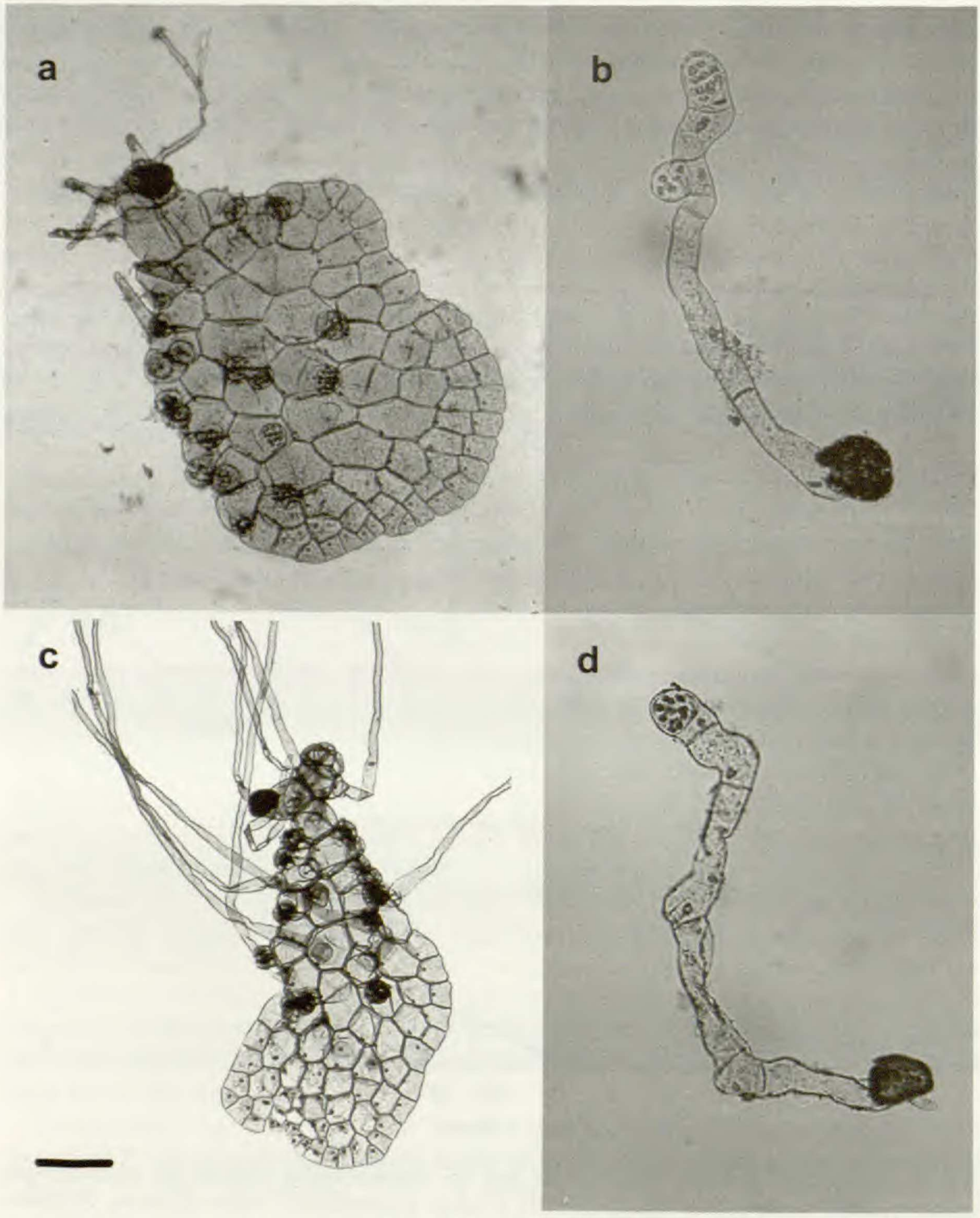

FIG. 4. a. Precocious male gametophyte of *P. vittata* VA, induced by female gametophyte of *P. incompleta* CA, two weeks after sowing; b. Antheridiate filamentous gametophyte of *P. vittata* VA produced in the dark six weeks after sowing in cultures with a female gametophyte of *P. incompleta* GC; c. Precocious male gametophyte of *P. incompleta* GO induced by female gametophyte of *P. vittata* VA; d. Antheridiate filamentous gametophyte of *P. incompleta* GO produced in the dark six weeks after sowing in cultures with a female gametophyte of *P. vittata* VA. Scale bar 50 μm.

Spore cultures of *P. vittata* VA grown in the dark and in the presence of female *P. incompleta* gametophytes (GC), showed a low percentage of spore germination (3%) 4 weeks after sowing. Gametophytes at this point consisted of filaments 2–3 cells long. Plates maintained in the dark for 6 weeks showed 7% germination and filamentous gametophytes, some of which were branched, and some bearing 1–2 antheridia (Fig. 4b). Similar gametophytes developed from spores of *P. incompleta* (GO) sown in the dark with a female gametophyte of *P. vittata* (Fig. 4d). In control cultures, those sown in the dark without female gametophytes, there was no germination.

Statistical significance of antheridiogen essays.—To check statistical significance of the results we performed chi-square analyses for each of the experiments (7 in total, Fig. 3). Data from control cultures were used as expected frequencies, while observed frequencies were obtained from cultures with female gametophytes. In all cases, $p < 0.05$ (d.f. = 7), therefore we accept that observed male precocity is due to the presence of female gametophyte. The only exception was for GC/GC experiment, in which, as is shown in Fig. 3b, development of antheridia was delayed in time with respect to its control culture.

Discussion

Gametophytes of *P. incompleta* follow the same developmental pattern described by Atkinson (1973) for *P. tremula* R. Br and *P. multifida* Poir., and form nearly symmetrical, cordate prothalli at a young stage. This is in contrast to other species of the genus, such as *P. berteroana* C. Agardh, *P. comans* Forst., *P. grandifolia* L., and *P. vittata*, that have a strongly asymmetrical shape at early stages of gametophyte development (Stokey and Atkinson, 1952; Mendoza *et al.*, 1996–97).

The most common sequence of sexual development in homosporous ferns involves the formation of antheridia followed by archegonia (Atkinson and Stokey, 1964). This sequence of gametangia development has been reported to be fixed for several taxa (Herrero *et al.*, 1993; Prada *et al.*, 1995). In the case of *P. incompleta* progression of sex expression was slightly different in the samples studied, and varied depending on the culture medium. On soil cultures, sample GC produced initially female gametophytes and later male ones, most of which ultimately became cordate and bisexual. Sample CA produced male and female gametophytes concurrently, which should favor intergametophytic crossing (Klekowsky and Lloyd, 1968), as well as a small proportion of other bisexual gametophytes in which antheridia were located on irregular lobes of some of the cordate females. Similar female gametophytes with antheridial lobes were reported in *Bommeria* (Haufler and Gastony, 1978), a genus in which the existence of an antheridiogen system has been demonstrated.

Agar cultures showed delayed gametangia differentiation with respect to soil cultures, and in the case of sample GC the sequence was different. A culture

medium influence on both aspects of sexual development (time of gametangia development and sequence of sexual expression) has also been shown in *Onoclea sensibilis* L. by Rubin and Paolillo (1983); in other taxa sexual sequence appears to be independent of substrate type (Haufler and Ranker, 1985).

Our assays to test antheridiogen activity demonstrate the existence of a functional antheridiogen system operating in *P. incompleta*, inducing both male precocity and germination in darkness. Female gametophytes of the different samples promote the rapid development of antheridia in young prothalli in all samples tested, except in the GC/GC paired cultures. However, the clearly positive results of the CA/GC test indicate that the antheridiogen system is present in *P. incompleta*. Nevertheless, it exhibits remarkable variation in response between our samples.

In regard to exogenous antheridiogen activity, our assays demonstrate the relationship between the species here studied: female gametophytes of *P. vittata* induce rapid male development in *P. incompleta*, and female gametophytes of various samples of *P. incompleta* induce rapid male precocity in *P. vittata*. But the responses of intrapopulational pairings in *P. incompleta* (GC/GC and GO/GO) show a decreased response when compared to either interspecific or interpopulational pairings. Our results on dark germination of *P. vittata* spores in the presence of female gametophytes of *P. incompleta* GC and the failure of control cultures to germinate in the dark also support the existence of an antheridiogen system in *P. incompleta*, which has the same effects on *P. vittata* as its own antheridiogen.

Variability in antheridiogen response among individuals and populations has been found in some species (Schneller *et al.*, 1990) and this appears to be the case in our study. However, since only one sporophyte per population has been studied, more assays are needed to determine to what extent the antheridiogen system of *P. incompleta* is effective in natural conditions and how it influences the reproductive biology of this species.

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